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MDMA-induced serotonergic neurotoxicity enhances aggressiveness in low- but not high-aggressive rats

Alinde E. Wallinga *, Anna M. ten Voorde, Sietse F. de Boer, Jaap M. Koolhaas, Bauke Buwalda

Department of Behavioral Physiology, Biological Center, University of Groningen, PO Box 14, 9750 AA Haren, the Netherlands

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ABSTRACT

Ecstasy or 3,4-methylenedioxymethamphetamine (MDMA) is a frequently (ab)used recreational drug for its acute euphoric effects but on the long-term may cause neurotoxic damage to serotonin (5-hydroxytryptamine; 5-HT) nerve endings in the brain. Since decreased brain 5-HT function has been strongly associated with several impulse control disorders like hostility and violent aggression, ecstasy users might be at risk developing this form of psychopathology. The present study examined the ability of a MDMA administration protocol (3×6 mg/kg, with 3 h intervals at 25 ºC ambient temperature), that previously was shown to partially deplete brain serotonin levels, to increase offensive aggressive behavior in male Wild-type Groningen (WTG) rats. This rat strain is known for its broad individual variation in offensive aggression. Resident-intruder aggression was assessed 5 days before and 23 days after MDMA administration. On day 28, MDMA neurotoxicity to 5-HT nerve terminals was assessed by quantification of serotonin reuptake transporter (SERT) immuno-positive axons in defined brain regions. Based on their expressed aggression level in the initial aggression test, rats were divided into low (<10% aggression), high (>50% aggression) or medium aggressive (10–50%) groups. The study demonstrated that MDMA treatment increased aggressiveness in only low aggressive rats and not in medium and high aggressive animals. Irrespective of their initial aggressiveness, MDMA significantly reduced the number of SERT-positive axons in all animals. In conclusion, vulnerability for increased aggression long after a single MDMA treatment is dependent on the individual's trait aggressiveness but not on the degree of MDMA-induced serotonergic neurotoxicity.

1. Introduction

Ecstasy or 3,4-methylenedioxymethamphetamine (MDMA), is a serotonin releaser that is frequently used for its acute euphoric effects, which include the feeling of more energy, increased confidence, elevated mood and affection (Liechti et al., 2001; Parrott, 2001; Vollenweider et al., 1998). Besides the acute MDMA toxicity risks (i.e., severe hyperthermia and renal failure), frequent use of this drug has given rise to concern since there is substantial evidence that MDMA can induce persistent negative neuropsychological effects.

Several studies investigating the long-term effects of MDMA on the serotonergic system in humans and animals have shown that MDMA can induce a lasting decrease in serotonin (5-hydroxytryptamine; 5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels in the brain, a reduction in the activity of tryptophan hydroxylase (TPH) and a reduction in the density of the serotonin transporter (Battaglia et al., 1987; Hewitt and Green, 1994; Schmidt and Taylor, 1987; Sharkey et al., 1991; Stone et al., 1986; Stone et al., 1987; Xie et al., 2006). Since decreased 5-HT levels have been associated with mood disorders including increased levels of impulsivity and aggression, ecstasy users might be at risk developing this form of psychopathology.

Studies investigating the effect of ecstasy use in humans indeed showed a lowering of mood starting several days after ecstasy use (Curran et al., 2004; Curran and Travill, 1997; Parrott and Lasky, 1998; Verheyden et al., 2002). Increased levels of hostility, impulsivity and aggression (Curran et al., 2004; Gerra et al., 2002; Hoshi et al., 2004; Hoshi et al., 2006; Morgan, 1998; Parrott et al., 2000; Verheyden et al., 2002) have been reported although also negative findings are present (Hoshi et al., 2007; McCann et al., 1994). This is consistent with the strong evidence that serotonin plays a crucial role in the regulation of aggressive behavior. Deficiencies in central serotonergic functioning are generally associated with increased levels of aggressive behavior in both humans and in rodents (Brown et al., 1982; Clark and Neumaier, 2001; Coccaro, 1989).

Surprisingly, only one study has investigated the long-term effects of MDMA on aggressive behavior in rats. No changes in aggressive behavior were seen in rats exposed to a single injection of MDMA 21 days earlier (Kirilly et al., 2006).

In the present study we examined the ability of repeated ("binge") dosing of MDMA (3×6 mg/kg, with 3 h intervals at 25 ºC ambient temperature) that is known to cause a long-term

* Corresponding author. Tel.: +31 503632337; fax: +31 503632331.
E-mail address: A.E.Wallinga@rug.nl (A.E. Wallinga).

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depletion of 5-HT in various brain regions (Sanchez et al. 2004), to increase offensive aggressive behavior in Wild-type Groningen rats (*Rattus norvegicus*). This rat strain was selected for our experiments because these animals are known to exhibit a rich repertoire of social behavior including aggressive behavior. Furthermore, they show a wide and consistent individual variation in aggressive behavior, in contrast to other laboratory rat strains where the display and individual variation of aggression is only poorly expressed (de Boer et al., 2003).

Individual variation in personality traits like impulsiveness/aggressiveness seems to be an important factor determining the individual's resilience or vulnerability to the neurotoxic and or psychopathological effects of psychoactive drugs like MDMA (Kelly et al., 2006; Semple et al., 2005; White et al., 2006). A recent human study indeed showed large inter-individual variation in MDMA-induced symptomatic neurotoxicity depending on their expressed personality traits (Reid et al., 2007). As the individual level of aggressiveness is causally related to a differential serotonergic neurotransmission (Koolhaas et al., 2007), we used this measure as a dependent variable of the MDMA-induced behavioral and neurotoxic effects.

2. Materials and methods

2.1. Subjects

This study has been approved by the animal experiments committee of the University of Groningen (DEC protocol # 4396F). Individual experiments were carried out in 5 different cohorts of animals over a period of 23 months using 21 animals in each experiment. At the moment of drug/vehicle treatment, male Wild-type Groningen (WTG) rats (*Rattus norvegicus*) weighed on average 425 ± 3.5 g. Room temperature was 21 °C, except during the time of MDMA/saline administration. Light:Dark cycle was 12:12 h (lights on at 01.00 h). Food (chow) and water were available ad libitum throughout the whole experiment. After weaning at the age of 23 days, rats were housed in groups of six males in clear Plexiglas cages (55 × 34 × 20 cm), until they were tested for offensive aggression in a standardized resident-intruder paradigm at an age of approximately 19 weeks. For this test, each rat was housed in a large cage (80 × 55 × 40 cm) together with a female to stimulate territorial behavior and prevent social isolation. One day before injections animals were individually housed in clear Plexiglas cages (38 × 22 × 15 cm) until 5 days before the last aggression test.

2.2. Aggression-test

After a week of habituation, four resident-intruder aggression tests were carried out on consecutive days as described previously (de Boer et al., 2003) in order to assess animals for the display of offensive aggressive behaviors against a male unfamiliar conspecific intruder. Before the start of each test, the female partner was removed (approx. 30 min in advance), and an unfamiliar male Wistar rat was introduced into the home cage of the experimental rat. The intruder Wistar rats weighed on average 350 g (4 months old) and were socially housed. Repeated resident-intruder test were performed to obtain a consistent characterization of offensive aggressive trait characteristics (de Boer et al., 2003).

During the first three tests only the attack latency time was scored, and the test was terminated shortly after occurrence of the attack or (in case of no attacks) after maximum test duration of ten min. During the fourth test the full behavioral profile was live recorded for ten min. by an experienced observer blind to the treatment condition. The duration of the following behavioral elements were scored: Offensive aggressive behavior (clinch, lateral threat, offensive upright, keep down, chase), social exploration (non-aggressive investigation of opponent), non-social behavior (explore environment, rear), social interaction (offensive behavior + social exploration), immobility and grooming (de Boer et al., 2003). 23 Days after the MDMA/saline injections, a similar resident-intruder aggression test was performed and the full behavioral profile was scored.

2.3. MDMA injections

Based on the percentage of time rats spent on offensive behavior during the initial aggression test before treatment, rats were categorized into three different groups, namely low aggressive (LA) (<10% offensive behavior), high aggressive (HA)(>50% offensive behavior) and medium aggressive (MA)(between 10%–50% offensive behavior) (Fig. 1A.). This trimodal categorization of aggressive phenotypes is chosen based on previous findings (de Boer et al., 2003). Within each aggression group half of the rats received MDMA treatment and the other half vehicle. MDMA treatment consisted of three times 6 mg/kg MDMA (±MDMA–HCl, 99,6% obtained from the Dutch Forensic Institute, The Netherlands), administered i.p. with 3 h interval (Sanchez et al., 2004), whereas the vehicle treatment consisted of three times saline (1 ml/kg, i.p.) injection. All injections were administered in the light phase, with the first injection given 1 h

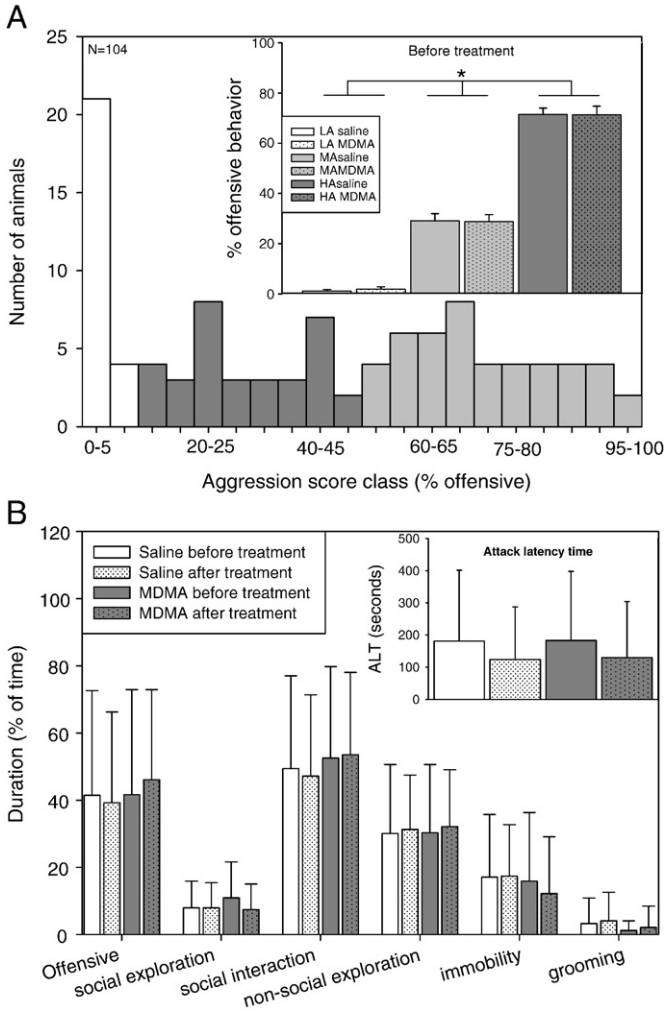

Fig. 1. A. Histogram showing the individual variation in levels of offensive aggressive behavior towards an unfamiliar intruder rat. Insert: Levels of offensive behavior (% duration (±S.E.M.) displayed during the first aggression test in low (LA), medium (MA) and high aggressive (HA) rats before receiving saline or MDMA treatment). *P<0.05. B. Ethogram of all rats in the resident intruder paradigm before and after receiving either MDMA (3 × 6 mg/kg) or saline (3 × 1 ml/kg) injections (Average ± s.d.).

after lights switched on. Ambient room temperature was temporarily raised to 25 °C 1.5 h before the first injection until 3 h after the last injection.

2.4. Brain processing and immunocytochemistry

Four weeks after the injections 63 rats (the first 3 batches) were sacrificed by transcardial perfusion (under terminal anaesthesia with pentobarbital) with heparinised saline followed by 2.5% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer. Brains were removed and after dehydration in 30% sucrose, the left hemisphere was cut into 40 µm sagittal sections on a cryostat microtome. For immunocytochemistry two brain regions known to be engaged in the regulation of aggressive behaviour were selected (hypothalamic attack region represented in the tuber cinereum (Hrabovszky et al., 2005) and the lateral orbital cortex (Halasz et al., 2006) and two brain regions (hippocampus and occipital cortex) more frequently studied in MDMA studies describing the effect of MDMA on 5-HT axons). Two sections containing CA3 region of the hippocampus, lateral orbital cortex and occipital cortex (sagittally lateral 2.40 mm according to Paxinos and Watson, 1998) and two sections containing tuber cinereum (sagittally lateral 0.90 mm) were selected. After pre-incubation with 5% normal horse serum and 0.4% triton-X100, sections underwent incubation with H_2O_2 to inactivate endogenous peroxidase. The primary antibody applied was mouse anti-SERT (1:1000, Biogenesis, Poole, United Kingdom; incubation at 4 °C for five days). As a second antibody biotinylated horse-anti-mouse (1:500, Vector BA2001) was used. After incubation with an ABC kit (1:500, Vector, Burlingame, United Kingdom) for 16 h, staining was visualized with diaminobenzidine and ammonium nickel sulphate (15 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB)/100 ml Tris–HCl, and 150 mg ammonium nickel sulphate /100 ml Tris–HCl) as chromogen.

2.5. Quantification

During the analysis of the brain material, the experimenter was blind to the treatment and aggression category of the rats. SERT immuno-positive axons were counted in the lateral orbital cortex, tuber cinereum (hypothalamus), occipital cortex and the CA3 region of the hippocampus with a 400x magnification using a grid (0.0625 mm^2, 100 squares). Axons that crossed the vertical and horizontal lines of every other line of the grid were counted. Quantification was performed independently by two different persons. There were no differences in the quantification between both persons.

2.6. Data analysis

SPSS 14.0 for Windows was employed to analyze the data statistically. Initially each behavioral category and attack latency time was analyzed by a repeated-measurements ANOVA with treatment (2 levels) and test as the within-subject repeated measurements factor. The difference in behavior of each behavioral category between the first and the second resident-intruder test was analyzed by a two-way ANOVA with treatment (2 levels) and aggression phenotype (3 levels) as between-subject factors. In case of significant main and/or interaction effects, post hoc analyses were performed using a one-way ANOVA or Student's *t*-test. The immunocytochemistry data were analyzed per brain area, using a two-way ANOVA, with treatment (2 levels) and aggression phenotype (3 levels) as between-subject factors. When a significant interaction was found, the effect of the treatment within the defined aggression groups was analyzed with a Student's *t*-test. The effect of aggression within the defined treatment groups was analyzed with a one-way ANOVA.

3. Results

3.1. Behaviour: individual differences in aggression and distributions of aggression scores

As commonly seen in our WTG rat strain, individual male resident rats differ widely in their level of species-typical offensive aggression expressed towards an unfamiliar intruder male, ranging from no overt aggression at all to very high levels of intense and incessant patterns of aggressive behaviour. Fig. 1A shows the distribution of all animals over the various offensive aggression score classes. Although all classes are represented, it is obvious that the individual behavioural scores are certainly not normally distributed (Shapiro–Wilkinson test for normality revealed significant deviation from a normal distribution). Based on the similarity of the previously described trimodal distribution pattern of aggressive phenotypes (de Boer et al., 2003), rats were categorized into low aggression (24% of the animals showing <10% offense), high aggressive (45% showing >50% offense) and medium aggressive (31 % of the animals showing between 10 and 50% offense) (Fig. 1A).

3.2. MDMA treatment

Three weeks after MDMA-treatment, no significant changes in the level of aggressiveness or any of the other scored behavioral indices (e.g., no main treatment effects for offensive behavior ($F(1,98) = 1.976$; $P = 0.163$) and latency to attack ($F(1,98) = 0.090$; $P = 0.765$) were noted in the total group ($n = 53$) of drug-treated animals as compared to either their pre-treatment offensiveness or the vehicle-treated group ($n = 51$)(Fig. 1B).

However, when the individual level of aggressiveness was taken into account, analyzing the changes in aggressive behavior in these rats after MDMA treatment, two-factor ANOVA testing significant interactions between treatment and aggressive phenotype only for offensive behavior ($F(2,98 = 3.197)$; $P = 0.045$). (In Table 1 the percentages of time spent on all behaviors during the first and the second resident-intruder aggression test is shown). As can be seen in Fig. 2, a pronounced increase in aggressiveness was observed after MDMA treatment in the low aggressive animals as compared to their own pre-treatment level of aggressive behavior ($t(11) = -4.916$; $P<0.001$) as well as in comparison to the vehicle-treated low-aggressive animals ($F(1,23) = 6.651$; $P = 0.017$). There were no significant effects of MDMA treatment on aggressive behavior (neither in attack latency time nor in total offensive behavior) in medium and high aggressive groups of rats. Surprisingly, irrespective of type of treatment, high aggressive animals showed significantly decreased levels of offensive aggression three weeks after the vehicle- or MDMA-treatment compared to their initial pre-treatment levels ($t(46) = 5.592$; $P<0.001$).

3.3. Immunocytochemistry

Four weeks after either vehicle or MDMA-treatment, the number of SERT-positive axons was determined. When individual levels of aggressiveness and treatment were taken into account, the two-factor ANOVA revealed no significant interaction for each of the measured brain regions. The analysis showed that MDMA treatment reduced the number of SERT-positive axons independent of the aggression phenotype (Main treatment effect) in CA3 region of the hippocampus ($F(1,51) = 27.580$; $P<0.001$), tuber cinereum ($F(1,39) = 4.881$; $P = 0.033$), Lateral orbital cortex ($F(1,55) = 15.333$, $P<0.001$), Occipital cortex ($F(1,53) = 12.054$; $P = 0.001$) (Fig. 3).

4. Discussion

The present study demonstrates that only individuals characterized by low trait-like aggressiveness show a robust increase in

Table 1
Percentage time spent on behavior during resident-intruder aggression test in low-aggressive (LA), medium aggressive (MA), and high-aggressive (HA) rats.

	Vehicle treated rats					
	1st aggression test			2nd aggression test		
	LA	MA	HA	LA	MA	HA
Offensive aggression	1.1 ± 0.6	29.1 ± 2.8	71.0 ± 2.6	14.7 ± 6.0	49.5.1 ± 6.0	46.8 ± 5.2
Social exploration	11.5 ± 1.6	11.4 ± 2.7	3.9 ± 0.8	13.6 ± 2.7	7.7 ± 1.8	5.1 ± 0.9
Non-social exploration	52.1 ± 6.9	31.5 ± 3.4	17.5 ± 1.7	37.5 ± 4.7	25.3 ± 2.8	30.1 ± 3.4
Immobility	30.7 ± 7.2	22.3 ± 3.5	6.2 ± 1.7	25.8 ± 5.7	15.7 ± 3.3	14.3 ± 2.7
Grooming	4.6 ± 2.5	5.4 ± 2.4	1.2 ± 1.0	8.4 ± 3.9	1.9 ± 1.1	3.6 ± 1.3
	MDMA treated rats					
	1st aggression test			2nd aggression test		
	LA	MA	HA	LA	MA	HA
Offensive aggression	1.8 ± 0.9	28.8 ± 2.8	69.4 ± 3.1	41.5 ± 8.3[a]	38.2 ± 6.5	51.5 ± 5.6
Social exploration	17.9 ± 4.4	14.7 ± 2.1	4.1 ± 0.7	10.0 ± 2.3	9.2 ± 1.8	5.4 ± 1.7
Non-social exploration	40.5 ± 7.0	39.8 ± 4.2	20.2 ± 2.9	35.1 ± 6.2	32.9 ± 3.2	31.6 ± 3.9
Immobility	36.9 ± 8.5	15.4 ± 2.8	5.9 ± 1.8	13.4 ± 8.1	15.5 ± 3.1	9.7 ± 2.6
Grooming	2.8 ± 1.4	1.3 ± 0.6	0.3 ± 0.3	0.3 ± 0.3	4.1 ± 2.7	1.8 ± 0.7

The first aggression test was 5 days prior to treatment with either vehicle or MDMA. The second aggression test was 23 days after administration of vehicle or MDMA.
Values are expressed as means ± S.E.M.
[a] $P<0.05$; MDMA 2nd aggression test versus Vehicle 1st aggression test.

aggressive behavior three weeks after MDMA administration. As MDMA treatment is known to produce long-term damage to serotonergic axons (Callahan et al., 2001; O'Hearn et al., 1988), an enhanced aggressiveness is not entirely unexpected and in accordance with the long-standing 5-HT deficiency hypothesis of aggression (Berman et al., 1997; Coccaro, 1989). This much-favoured hypothesis proposes that basal measurements of brain 5-HT activity are inversely correlated with indices of aggression and impulsivity leading to the suggestion that reduced 5-HT activity might be an important factor contributing to the expression of heightened aggressiveness (Miczek et al., 2004). Such a link between reduced central serotonergic function and increased aggressiveness is indeed supported by many (Buchanan et al., 1994; Chiavegatto et al., 2001; Gianutsos and Lal, 1975; Gibbons et al., 1978; Vergnes et al., 1986) but not all (De Almeida et al., 2001; Sijbesma et al., 1991) pharmacological brain serotonin depletion studies in animals.

Surprisingly, however, no effect of MDMA-treatment on levels of aggressive behavior was seen in medium and high aggressive rats. To exclude the possibility that in high and medium aggressive rats no behavioral change was found because the employed MDMA regimen did not lead to serotonergic fibre loss or depletion in these rats, the serotonergic neurotoxic profile in all rats was assessed. Immunochemical quantification of SERT positive axons is known to be a reliable method to determine MDMA-induced serotonergic neurotoxicity (Xie et al., 2006). The data revealed that our MDMA administration regimen resulted in a similar level of serotonergic neurotoxicity in all MDMA treated animals irrespective of their aggressive phenotype. Thus, although serotonergic deficiencies are generally implicated in the enhancement of offensive aggressive behavior, only low aggressive rats exhibit increased levels of aggression after the MDMA-induced 5-HT depletion.

The question why only low and not medium and high aggressive rats increase their aggressiveness after MDMA-induced 5-HT neurotoxicity is not easy to answer. The differences between the three groups of rats cannot be explained by a ceiling effect in the high aggressive males since medium aggressive rats also do not change their levels of aggression after MDMA treatment. It seems more likely that the results may reflect true individual differences in the response to MDMA and the underlying physiological mechanisms. Our data showing different individual changes in aggressive behavior are consistent with the differential response of high, medium and low aggressive rats to 5-HT_{1A} antagonists and selective serotonin reuptake

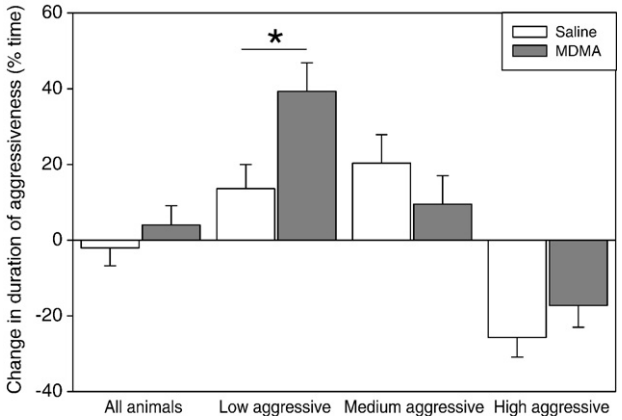

Fig. 2. Change in the levels of offensive aggressive behavior (% duration ± S.E.M) as displayed in low, medium and high aggressive rats that received either saline or MDMA treatment 21 days earlier. *$P<0.05$.

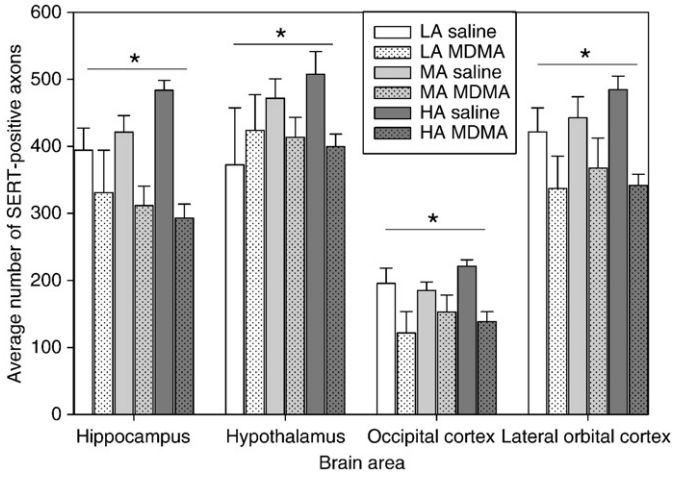

Fig. 3. Average numbers of SERT-positive axons (±S.E.M.) in lateral orbital cortex, occipital hippocampus and hypothalamus in low (LA), medium (MA) and high aggressive (HA) rats 28 days after saline or MDMA injections. * represents the significant differences ($P<0.05$) between the different treatments, independent of aggression type.

inhibitors (SSRI's). High and low aggressive males show opposite dose–response relationships in response to the serotonin 5-HT_{1A} receptor antagonists and SSRI's with no response in the intermediate males (de Boer et al., 2001). However, in the present study possible anti- or pro-aggressive effects of MDMA in the high aggressive males may be obscured by the unusual decrease in aggression of saline treated rats, observed in the second aggression test. This decrease was unexpected since several prior longitudinal studies in our lab using these WTG rats have repeatedly demonstrated that the individual level of aggressiveness is rather consistent through time. The only difference between this study and many of these prior studies is the employed drug/vehicle treatment schedule under high ambient temperature. Perhaps high aggressive animals are more sensitive to this stressful event in that it causes a persistent change in their tendency to behave aggressively.

Considering the other measured behavioral parameters, changes in immobility levels are the most interesting to focus on. Our experiment showed that before MDMA/saline treatment, the three aggression categories differed also significantly in their levels of immobility behavior. Our main result that only low trait-like aggressive individuals are vulnerable for developing 5-HT related changes in aggressive behaviour after MDMA administration support the recent observation in humans that individuals with high self-control become more aggressive whereas aggressive behaviour of individuals with low self-control changes little (Reid et al., 2007). We would like to stress the importance of this finding because it demonstrates the necessity to take individual variation into account in studies investigating the behavioral changes after MDMA (ab)use.

In conclusion, our results clearly show that the vulnerability for increased aggression long after a single MDMA treatment is dependent on the individual's trait aggressiveness and not on the degree of MDMA-induced serotonergic neurotoxicity. This indicates that only low trait-like aggressive individuals are vulnerable for developing 5-HT related changes in aggressive behaviour after MDMA administration. It is tempting to speculate that these individuals might also be more at risk of developing 5-HT related psychopathologies, including pathological forms of aggressive behavior.

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